REMARKS

Reconsideration of this application, as amended, is respectfully requested.

The Examiner made the restriction requirement final but kindly modified the groups by rejoining Group II (Claim 3 in part) with Group I (Claims 1, 2, 6 in part and 8-13) and permitting rejoinder of Groups XIII (Claims 14-16) and XIV (Claims 19 and 20) if the claims are amended to depend on the product claims of Group I or otherwise include their limitations. Nonelected Claims 3 in part, 4, 5, 7 and 14-20 are withdrawn from consideration. (The Examiner had included Claims 2 and 21 as withdrawn claims but it is noted that Claim 2 is included in the elected Group I, and Claim 21 does not exist in the application. Also, the nonelected subject matter (*i.e.*, protein and immunogenic fragment) of Claims 6 in part and 8 in part should have been withdrawn from consideration under the Examiner's restriction requirement.)

In response to the final restriction requirement, the present amendment confirms the status of Claims 4, 5, 7, 17 and 18 as being withdrawn from consideration in this patent application, removes the nonelected subject matter from Claims 3, 6 and 8-13, and amends Claims 14, 15, 19 and 20 to include the limitation of the product claims of Group I. Claims 2 and 16 have been cancelled. Applicants reiterate their right to file a divisional application directed to the nonelected subject matter of this invention in due course.

The amendment takes care of the Examiner's objection to Claims 3 and 6 as reciting nonelected inventions. Although the Examiner did not formally object to Claim 8, Applicants have omitted the nonelected protein and immunogenic fragment from Claim 8 to advance prosecution towards an early allowance.

The Examiner rejects Claims 1-3, 6 and 8-13 (now Claims 1, 3, 6 and 8-13) under 35 U.S.C. § 112, second paragraph, since the Examiner believes the language is indefinite for reasons set forth in the Office action on pages 4 and 5. To expedite matters, Applicants have rewritten the claims for the better readability thereof. Since the amendment will render the rejection moot, Applicants respectfully request that this rejection be withdrawn.

The Examiner also rejects Claim 1 under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement for reasons set forth in the Office action on pages 5-6. To expedite matters and without comment as to the merits of the rejection, Applicants have amended Claim 1 to conform to the allowable subject matter of the claimed invention. It is

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believed that the amendment will obviate the rejection and, hence, this rejection should be withdrawn.

The Examiner rejects Claims 8-13 under 35 U.S.C. § 112, first paragraph, as failing to provide enablement for a vaccine comprising an isolated avian hepatitis E virus ("HEV") that confers protection against a viral infection or disease, or for a method of vaccination using such a vaccine for reasons set forth in the Office action on pages 7-9. Applicants respectfully traverse the rejection.

Enablement requires that one of ordinary skill in the art would be able to practice the claimed invention without an undue amount of experimentation. A determination of what amount of experimentation would constitute an undue amount must apply a standard of reasonableness. In fact, a considerable amount of experimentation is permissible as long as it is merely routine effort and the specification has provided a reasonable amount of guidance. To satisfy the statutory requirement, it is well established, however, that the specification does not need to contain any working examples and does not need to convince persons skilled in the art that the assertions in the specification are correct.

Applicants' disclosure is presumed to be accurate unless there is reason to doubt the objective truth of the statements regarding the vaccine formulations. The Examiner has cited Fields VIROLOGY (1996) to explain why she doubts the truth or accuracy of Applicants' assertions in the application. On close examination of the Examiner's evidence, however, it is found that the selected passages have been taken out of context and do not support her position. The disadvantages of nonliving virus vaccines cited on pages 480-482 mainly refer to problems identified many years ago with formalin-inactivated measles virus and RSV vaccines. Lessons were learned on how to remedy the problem. Be that as it may, there are certain disadvantages of inactivated vaccines in relation to live attenuated viruses that have been well documented. On the other hand, as Fields describes on page 480, there are equally well-documented advantages to nonliving virus vaccines that make preparation and clinical trials worthwhile. Thus, the publication simply teaches that nonliving virus vaccines can be made and tested by standard techniques to determine their potential disadvantages and advantages. It is a mere matter of routine experimentation, therefore, to make the inactivated avian HEV vaccine and determine whether any disadvantages outweighs its benefits.

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As proof of genetic roulette, the Examiner cites the single failure of Theiler to make additional attenuated mutants of yellow fever after successfully obtaining an attenuated 17D strain of virus and adds that the genetic basis for attenuation of measles, mumps, rubella, yellow fever and vaccinia viruses is unknown. Theiler's failure in light of his earlier success and the unknown basis for attenuation of several viruses are totally irrelevant to enablement in the present case particularly since vaccines against measles, mumps, rubella, yellow fever and vaccinia viruses have been successfully prepared and used in the past. The fact that they may not have been characterized as extensively as poliovirus vaccine does not lessen their viability or usefulness as vaccines. Indeed, one of ordinary skill in the art does not need to know the mechanism of action or the genetic basis of attenuation in order to make and use a viral vaccine without undue experimentation.

In further support of enablement, Fields refers to the successful Jennerian approach on page 488 to developing live attenuated viruses such as bovine parainfluenza virus type 3 and rhesus rotavirus, which is being studied as a vaccine candidate. The Jennerian approach uses a virus strain of mammalian or avian origin to immunize humans against a human virus that is related antigenically to the animal or avian strain. In the present application, Applicants teach that the vaccine based on the avian HEV can be preferentially designed to protect against human hepatitis E through this so-called Jennerian approach, *i.e.*, the approach taken by Edward Jenner to develop the cowpox virus vaccine useful against human smallpox (see the sentence bridging pages 23-24).

Contrary to the Examiner's belief that the specification does not teach that any avian HEV vaccine is protective against an avian or mammalian HEV infection or has any immunogenic properties, Applicants would like to point out that the working examples demonstrate that avian HEV is antigenically related to Sar-55 human HEV and swine HEV as well as chicken Big Liver and Spleen Disease Virus ("BLSV") (see the paragraph bridging pages 15 and 16, and Example 18 on pages 52 and 53). Western blot analyses revealed that antiserum to each virus strongly reacted with homologous antigen. It was also demonstrated that the antiserum against BLSV reacted with the recombinant ORF2 protein of avian HEV, indicating that BLSV is antigenically related to avian HEV (see page 16, lines 3-7). The antigenic relatedness of avian HEV ORF2 capsid protein with human HEV, swine HEV and chicken BLSV establishes that immunization

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with an avian HEV vaccine (either an attenuated or a recombinant vaccine) will protect not only against avian HEV infection, hepatitis-splenomegaly syndrome and BLSV infection in chickens but also against human and swine HEV infections in humans and swine. Thus, a vaccine based on avian HEV, its nucleic acid and the proteins encoded by the nucleic acid will possess broad spectrum, immunogenic activity against avian, swine and human HEVs, and BLSV. Fields substantiates that there is a reasonable basis under the Jennerian approach to believe that the claim-recited HEV vaccines can be made and used successfully in the method of the present invention.

The Examiner has further stated that the specification does not teach how to modify, inactivate or attenuate an avian HEV to produce an effective vaccine for chickens. Applicants disagree and assert that the amount of guidance in the application is sufficient in view of the current state of the art. The Examiner's attention is respectfully drawn to the numerous vaccine formulations and methods of administration that are described in detail in the application from page 19, line 6, to page 25, line 2. Of course, the Examiner will not find Applicants' avian HEV and the specific vaccines based thereon in the prior art since Applicants have made the first discovery of the novel avian HEV. Nevertheless, it is well within the ordinary skill of the art to make and use the avian HEV vaccine of the present invention. As the Examiner is aware, a recombinant subunit vaccine for human HEV is being developed. Vaccines against hepatitis A are also available in both the inactivated and live attenuated forms. Further support of enablement can be seen in the attached Review article of Liljeqvist *et al.*, "Production of recombinant subunit vaccines: protein immunogens, live delivery systems and nucleic acid vaccines," Journal of Biotechnology, 73:1-33 (1999), which describes the state of the art for nucleic acid vaccines on pages 15-19. In sum, it is clear that one of ordinary skill in the art can apply the direction provided in the instant specification to the current knowledge of viral vaccines and be able to make and use the claim-recited vaccines without undue experimentation. Nothing more is required by statute.

In view of the foregoing comments and the proffered evidence, it is respectfully asked that the rejection of Claims 8-13 under 35 U.S.C. § 112, first paragraph, be withdrawn.

The Examiner rejects Claim 1 under 35 U.S.C. § 102(a) as anticipated by or, in the alternative, under 35 U.S.C. § 103(a) as obvious over Shivaprasad *et al.* To expedite matters but

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without comment on the merits of this rejection, Applicants have amended Claim 1 to conform to the allowable subject matter of the claimed invention. It is believed that the amendment will obviate the rejection and, hence, this rejection should be withdrawn.

The Examiner has kindly indicated that SEQ ID NO:1 is free of the prior art and, as such, product claims limited to a polynucleotide comprising SEQ ID NO:1 and an isolated virus comprising this polynucleotide would be allowable. Most of the pending claims match the allowable subject matter. Insofar as Claims 8-13 are concerned, it is hoped that the Examiner will reconsider these rejected claims in a favorable light and allow the claimed subject matter.

Accordingly, it is believed that this application is now in condition for an allowance and such treatment is respectfully urged.

Respectfully submitted,
VIRGINIA TECH INTELLECTUAL PROPERTIES, INC.

Date: December 26, 2003

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CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8

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APPENDIX

AMENDMENTS TO THE CLAIMS

Please amend the claims as follows:

1 (Currently amended). An isolated avian hepatitis E virus having ~~the nucleotide sequence set forth in SEQ ID NO:1 or its complementary strand no more than about 80% nucleotide sequence identity to an Australian big liver and spleen disease virus~~.

2 (Cancelled).

3 (Currently amended). An isolated polynucleotide comprising a member selected from the group consisting of:

(a) ~~a~~ the nucleotide sequence set forth in SEQ ID NO:1 or its complementary strand; and

(b) ~~a~~ the polynucleotide which hybridizes to and which is at least 95% complementary to the nucleotide sequence set forth in SEQ ID NO:1; ~~and~~

~~(c) an immunogenic fragment selected from the group consisting of a nucleotide sequence in the partial helicase gene of ORF1 set forth in SEQ ID NO:3, a nucleotide sequence in the RdRp gene set forth in SEQ ID NO:5, a nucleotide sequence in the ORF2 gene set forth in SEQ ID NO:7, a nucleotide sequence in the ORF3 gene set forth in SEQ ID NO:9 and their complementary strands.~~

4 (Withdrawn).

5 (Withdrawn).

6 (Currently amended). An immunogenic composition comprising a nontoxic, physiologically acceptable carrier and an isolated avian hepatitis E virus having ~~a~~ the nucleotide sequence set forth in SEQ ID NO:1, its complementary strand; ~~or an~~ the isolated polynucleotide according to Claim 3 ~~or an antigenic protein encoded by the isolated polynucleotide~~.

7 (Withdrawn).

8 (Currently amended). A vaccine that protects an avian or mammalian species from viral infection or hepatitis-splenomegaly syndrome caused by an avian or mammalian hepatitis E virus comprising a nontoxic, physiologically acceptable carrier and a member selected from the group consisting of:

(a) a modified live avian hepatitis E virus, having ~~a~~ the nucleotide sequence set forth in SEQ ID NO:1 or its complementary strand;

(b) an inactivated avian hepatitis E virus, having ~~a~~ the nucleotide sequence set forth in SEQ ID NO:1 or its complementary strand;

(c) an attenuated avian hepatitis E virus, having ~~a~~ the nucleotide sequence set forth in SEQ ID NO:1 or its complementary strand; and

(d) an antigenic subunit of avian hepatitis E virus, having ~~a~~ the nucleotide sequence set forth in SEQ ID NO:1 or its complementary strand; and

(e) ~~a purified, immunogenic protein encoded by an isolated polynucleotide comprising a nucleotide sequence set forth in SEQ ID NO:1 or its complementary strand;~~ a polynucleotide which hybridizes to and which is at least 95% complementary to the nucleotide sequence set forth in SEQ ID NO:1. ~~; or an immunogenic fragment selected from the group consisting of a nucleotide sequence in the partial helicase gene of ORF1 set forth in SEQ ID NO:3, a nucleotide sequence in the RdRp gene set forth in SEQ ID NO:5, a nucleotide sequence in the ORF2 gene set forth in SEQ ID NO:7, a nucleotide sequence in the ORF3 gene set forth in SEQ ID NO:9 and their complementary strands.~~

9 (Original). The vaccine according to Claim 8, wherein said virus is inactivated or attenuated by serial passage of the virus through embryonated chicken eggs.

10 (Original). The vaccine according to Claim 8, wherein said vaccine further contains an adjuvant.

11 (Original). A method of protecting an avian or mammalian species from viral infection or hepatitis-splenomegaly syndrome caused by the avian or mammalian hepatitis E virus comprising administering an immunologically effective amount of the vaccine according to Claim 8 to an avian or mammalian species in need of protection against said infection or syndrome.

12 (Original). The method according to Claim 11, wherein the vaccine is administered to a chicken, a pig or a human.

13 (Original). The method according to Claim 11, wherein the vaccine is administered orally, intrabuccally, intranasally, transdermally or parenterally.

14 (Currently amended). A method for propagating, inactivating or attenuating a hepatitis E virus having the nucleotide sequence set forth in SEQ ID NO:1 or its complementary

strand comprising inoculating an embryonated chicken egg with a live, pathogenic hepatitis E virus and recovering ~~a~~ the live, pathogenic hepatitis E virus or serially passing the pathogenic virus through additional embryonated chicken eggs until said virus is rendered inactivated or attenuated.

15 (Original). The method according to Claim 14, wherein the live, pathogenic hepatitis E virus is injected intravenously into the embryonated chicken egg.

16 (Cancelled).

17 (Withdrawn).

18 (Withdrawn).

19 (Currently amended). A method for detecting an avian hepatitis E viral nucleic acid sequence having the nucleotide sequence set forth in SEQ ID NO:1 or its complementary strand in an avian or mammalian species comprising isolating nucleic acid from the avian or mammalian species, hybridizing the nucleic acid and determining the presence or absence of a hybridized probe complex.

20 (Original). The method according to Claim 19, wherein the nucleic acid is hybridized with a radio-labeled or a non-radiolabeled nucleic acid probe derived from the nucleotide sequence set forth in SEQ ID NO:1 or hybridized with a pair of oligonucleotide primers derived from the nucleotide sequence set forth in SEQ ID NO:1 and further amplified in a polymerase chain reaction.

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Review article

Production of recombinant subunit vaccines: protein immunogens, live delivery systems and nucleic acid vaccines

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Abstract

The first scientific attempts to control an infectious disease can be attributed to Edward Jenner, who, in 1796 inoculated an 8-year-old boy with cowpox (vaccinia), giving the boy protection against subsequent challenge with virulent smallpox. Thanks to the successful development of vaccines, many major diseases, such as diphtheria, poliomyelitis and measles, are nowadays kept under control, and in the case of smallpox, the dream of eradication has been fulfilled. Yet, there is a growing need for improvements of existing vaccines in terms of increased efficacy and improved safety, besides the development of completely new vaccines. Better technological possibilities, combined with increased knowledge in related fields, such as immunology and molecular biology, allow for new vaccination strategies. Besides the classical whole-cell vaccines, consisting of killed or attenuated pathogens, new vaccines based on the subunit principle, have been developed, e.g. the Hepatitis B surface protein vaccine and the *Haemophilus influenzae* type b vaccine. Recombinant techniques are now dominating in the strive for an ideal vaccine, being safe and cheap, heat-stable and easy to administer, preferably single-dose, and capable of inducing broad immune response with life-long memory both in adults and in infants. This review will describe different recombinant approaches used in the development of novel subunit vaccines, including design and production of protein immunogens, the development of live delivery systems and the state-of-the-art for nucleic acids vaccines.

Keywords: Vaccine development; Recombinant; Protein vaccine; Surface-display; Live bacteria; Viral vector; DNA vaccine; RNA vaccine

1. Introduction

Vaccination is one of the most important and cost-effective methods of preventing infectious diseases. Owing to world-wide vaccination programs, the incidence of many fatal diseases has

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drastically decreased. Most of the vaccines used routinely today as part of childhood immunisation programs are whole-organism vaccines (Table 1), consisting of live attenuated vaccines, or killed whole bacteria or viruses (Plotkin, 1993). Live attenuated vaccines are often able to induce strong, long-lasting immunity, cell-mediated as well as humoral. However, there exists a risk of reversion to virulent wild-type strains which can lead to disease when using attenuated bacteria or virus, especially in immunocompromised hosts. Killed vaccines, on the other hand, cannot replicate and are therefore non-infectious, but are less powerful than live vaccines in inducing protective immunity. Booster injections and the addition of adjuvant systems to improve immunogenicity are often needed with killed whole-cell vaccines. Another drawback with attenuated or killed whole-cell vaccines is that the existing requirements from regulatory authorities, e.g. the Food and Drug Administration (FDA) and the World Health Organization (WHO), for exact specifications of the vaccine composition and mechanisms to obtain immunity, are difficult to meet.

Bacterial polysaccharides and viral surface proteins, purified from pathogenic organisms, and detoxified toxins, are examples of subunit immunogens used and investigated as vaccines or vaccine components. The subunits of pathogenic origin are safe to use as vaccines, provided that the extraction procedure or detoxifying method gives a pure product. Yet, the production of such vaccines generally requires large-scale cultivation of pathogenic organisms, which often is very costly and not without risk. These type of non-recombinant subunit vaccines, consisting of single proteins or oligosaccharide vaccines, often need adjuvants or various conjugates to render them more immunogenic.

Table 1
Classification of widely used vaccines

Category of vaccine	Example
Whole cell	
Attenuated bacteria or viruses	Bacille Calmette-Guérin (BCG) (tuberculosis)
	Measles
	Mumps
	Rubella
	Oral polio vaccine (Sabin)
Killed bacteria and viruses	Pertussis
	Inactivated polio vaccine (Salk)
Subunit	
Toxoid	Diphtheria
	Tetanus
Capsular polysaccharide	*H. influenzae* type B
Yeast recombinant subunit	Hepatitis B surface protein

In 1986, the first recombinant subunit vaccine, the Hepatitis B surface antigen vaccine, produced in *Saccharomyces cerevisiae* (Valenzuela et al., 1982), was licensed. The basic principle for a subunit vaccine is that the gene encoding the subunit vaccine is isolated and transferred to a second, normally non-pathogenic organism. The recombinant subunit vaccine is then produced by the heterologous host, and can be designed to be delivered, either as a purified immunogen, or by using the production host as a live vector, or as pure nucleic acids in the form of a gene encoding the immunogen (Table 2). Advantages of recombinant subunit vaccines are numerous. First of all, the pathogen can be entirely excluded from the production of the vaccine, which eliminates risks associated with the production, as well as risks for contamination with toxic compounds, risks of reversion to virulent genotypes or incomplete inactivation of whole-cell vaccines. By optimising the delivery system, the immune responses can be tailored for the specific pathogen against which the vaccination is aimed, and many recombinant subunit vaccines are investigated for mucosal delivery, using appropriate vectors, e.g. live bacteria or virus, killed organisms, particulate delivery systems, or even transgenic plants.

The recombinant subunit approaches are today being investigated in the development of vaccines against organisms, for which no vaccine exists, and also in the search for new more effective vaccines with less adverse effects than the ones already on the market. Continuous attempts to improve on existing vaccines are made as knowledge in related fields such as immune mechanisms,

Table 2
Recombinant subunit vaccines and some of their characteristics

Recombinant vaccine	Typical characteristics
Protein immunogens	Defined composition. Safe. Induces primarily humoral immunity. Need for adjuvants. Cost depends on production system.
Live vectors: bacterial	Attenuated pathogens or food-grade/commensal bacteria. Possible oral vaccines. Humoral and cellular immunity. Surface display of antigenic determinants possible. A variety of delivery systems exists.
Live vectors: viral	Humoral and cellular immunity. Could be used for large or multiple immunogens. Risk for reversion into virulence through genetic recombination when using attenuated pathogens as carriers.
Nucleic acids: DNA	Cost-efficient production. Stimulates cellular and humoral immune responses. Inefficient transfection. Risk of integration into host genome not completely excluded. Possible bacterial delivery. In vivo amplification systems available.
Nucleic acids: RNA	Unstable. No risk of integration into host genome. Do not have to enter the nucleus for translation. In vivo amplification systems available.

bacterial pathogenesis, and genetics, increases. Genetically attenuated *Salmonella typhi* strains are currently being investigated in human trials as alternatives to the licensed oral vaccine against typhoid fever, *S. typhi* Ty21a, which carries non-defined mutations and requires multiple doses (Nardelli-Haefliger et al., 1996).

Many recombinantly produced vaccines, which have given promising results in preclinical and clinical trials (see Anon., 1995 for an overview), could be expected on the market in a few years' time. The most common recombinant strategies for the development of subunit vaccines will be described below.

2. Recombinant subunit vaccines

2.1. Recombinant protein immunogens

Molecular biology and genetic engineering have provided vaccine development with valuable tools for recombinant protein production, which enables single proteins to be easily produced in various hosts, with multiple possibilities to purpose-design the protein product and also the production process (Koths, 1995). In fact, a whole battery of strategies exists for optimising the production of a recombinant protein (Makrides, 1996). For example, when expressing the gene for an antigen in a production host, appropriate promoters can increase the production levels (Suarez et al., 1997), and signal sequences can target the recombinant protein to the secretory machinery for exportation, leading to facilitated purification of the product (Moks et al., 1987; Hansson et al., 1994). One of the most common problems encountered is proteolytic degradation of the heterologous protein. Today, a number of strategies can be evaluated in order to minimize proteolysis (Murby et al., 1996). Recombinant strategies that have been employed to simplify production of protein subunit vaccines include: (i) de novo synthesis of the gene fragment to be expressed (Murby et al., 1995); (ii) the production of only immunodominant subfragments of a target immunogen (Sjölander et al., 1993; Murby et al., 1994; Power et al., 1997); (iii) the use of fusion proteins to improve immunogenicity (Sjölander et al., 1997; Libon et al., 1999); (iv) protein engineering to improve solubility and stability (Murby et al., 1995); and (v) recombinant subunit immunogens can be adapted for direct adjuvant incorporation (Andersson et al., 1998). As will be discussed below, the choice of host offers further options for the design and production of the immunogen. Concerning the bioprocesses for production of recombinant subunit vaccines for clinical trials, the same rules apply as for the production of protein therapeutics in terms of quality control, characterization and specification of the product and requirements for validated good-manufacturing process (GMP) production. Therefore, an aim exists to base the process on the

least complicated host that produces an active subunit vaccine.

2.2. Synthetic peptides

Chemically synthesized peptides are excellent tools in vaccine research since they are extensively used for definition of T- and/or B-cell epitopes. However, synthetic peptides are also investigated as experimental vaccines. The only candidate malaria vaccine tested in large double-blind placebo-controlled trials in three continents, is in fact a polymerized synthetic peptide (Patarroyo et al., 1988). Peptides, identified as immunogenic epitopes, can elicit a strong immune response when delivered together with a carrier (Simard et al., 1997) or an adjuvant (Hsu et al., 1996b), but immunized without carrier or adjuvant, they are generally not very immunogenic since they are rapidly cleared in vivo (Ben-Yedidia and Arnon, 1997). Peptides could be chosen to stimulate predominantly either the humoral or cellular branch of the immune response. A single epitope-based vaccine will however not likely be effective in a broad out-bred population since the cellular response in man is restricted to special human leukocyte antigens (HLAs). The use of multiple epitope vaccines, such as the multiple antigen peptides (Tam, 1996), could potentially overcome this restriction. The modification of synthetic peptides derived from the HIV-1 envelope by a lipidic amino acid resulted in lipopeptides capable of inducing specific cytotoxic T lymphocytes (CTLs) after immunization of mice, without any adjuvant (Deprez et al., 1996). Since the lipopeptides can be produced by conventional synthesis methods at low cost, they might be suitable as vaccine candidates when a CTL response is of importance for protection. Retro-inverso peptides might have a potential as synthetic vaccines, as their reversed peptide bonds have better resistance to proteolytic degradation. Retro-inverso peptides have been shown to elicit T-cell responses upon immunisation of mice (Mézière et al., 1997), and to be superior to the corresponding L-peptide in eliciting antibody responses (Briand et al., 1997). A major drawback in the use of synthetic peptides is their limit in length. Synthetic peptides should normally be less than 50 amino acid residues so that they can be manufactured cost-efficiently. Since short synthetic peptides have a high degree of structural flexibility, and thus most probably would react with a wide spectrum of B-cells upon immunization, they are not particularly suited as subunit vaccines in cases where the humoral part of the immune response would be of importance for protection. In such cases, a more extended amino acid sequence, expressed by recombinant means, would have a greater probability of obtaining secondary structure elements and potentially a correct folding, for the display of immunodominant B-cell epitopes. A correct three-dimensional structure would obviously be of importance in order to elicit antibodies capable of recognizing the pathogen upon infection.

2.3. Passive vaccination strategies

Passive vaccination strategies, meaning immunizing with antibodies or antibody fragments, have been studied extensively both with anti-infectious (Ma et al., 1987, 1990; de Alboran et al., 1995) and anti-inflammatory (Elliott et al., 1994a,b) applications. Functional antibodies or fragments thereof have been recombinantly expressed in bacteria (Plückthun, 1992), yeast (Ridder et al., 1995), plants (Ma et al., 1995), and mammalian cells (Trill et al., 1995), and antibody design has been a further research focus (Winter and Milstein, 1991; Chester and Hawkins, 1995; Hayden et al., 1997), e.g. in creating humanised monoclonal antibodies (mAbs) (Hurle and Gross, 1994; Owens and Young, 1994). An alternative to the humanisation of mAbs is the use of combinatorial antibody libraries expressed on phages, from which high-affinity Fab fragments can be selected (McCafferty et al., 1990; Griffiths et al., 1994; Malmborg et al., 1996; Hoogenboom, 1997). Intranasal immunisation with Fab specific for respiratory syncytial virus (RSV) selected from a phage display library, resulted in significant reduction of virus titer in mice (Crowe et al., 1994, 1997). A number of recombinant antibody fragments and humanized monoclonal antibodies are today being investigated in human clinical trials in passive vaccination applications. Another

application for antibodies in vaccination studies, is the anti-idiotype antibody, having an antigen binding site resembling the antigen of interest (Dalgleish and Kennedy, 1988). Immunisation with anti-idiotype antibodies has conferred protection in animal disease models but no vaccine based on this principle has yet been licensed.

2.4. Production hosts

The recombinant hepatitis B virus (HBV) vaccine sold today is produced in *S. cerevisiae* (Valenzuela et al., 1982), but many other hosts are being investigated for recombinant antigen production. In Table 3 are listed some of the prevalent hosts for recombinant protein production and their main characteristics. Each production host offers numerous advantages for the recombinant antigen to be produced, but there are also limitations, which have to be considered when choosing a host.

E. coli, the number one bacterium of recombinant DNA technology, has been extensively studied as production host for heterologous proteins. Since it is very well characterised, many strategies for optimising protein expression and protein quality, e.g. choice of *E. coli* strain, transcriptional and translational regulation, and protein targeting to different cellular compartments, have been reported (see Makrides, 1996; Murby et al., 1996; Weickert et al., 1996; Hannig and Makrides, 1998 for reviews). Engineering of the recombinant protein itself can enhance the product quality and yield when produced in *E. coli*, as demonstrated for an RSV major glycoprotein fragment by Murby et al. (1995).

Bacteria, other than *E. coli*, have also been investigated for production of recombinant antigens and could sometimes be preferred with respect to proteases and components of the expression systems (Billman-Jacobe, 1996). *Salmonella typhimurium* (Martin-Gallardo et al., 1993; Liljeqvist et al., 1996), *Vibrio cholerae* (Viret et al., 1996), and *Bacillus brevis* (Ichikawa

Table 3
Hosts for the production of recombinant proteins and some of their characteristics

Host	Typical characteristics	References
Bacteria	Well characterized. Many genetic tools available. High production yields. Cost-efficient production. *E. coli* the dominating bacterium. No posttranslational modifications.	Billman-Jacobe, 1996; Makrides, 1996
Yeast	Well characterized. Various techniques for manipulating genes. Cost-efficient production. Post-translational modifications, except glycosylations, similar to higher eukaryotes. *S. cerevisiae* dominating. Higher yields possible for *Pichia pastoris*.	Sudbery, 1996
Insect cells	Bacoluvirus and plasmid-based expression systems available. Cheaper than mammalian cell lines. Large-scale production possible. Virus infection could impair insect cells, leading to low yields. Glycobiology differs from mammalian cells.	McCarroll and King, 1997; Possee, 1997
Plant cells	Expensive and costly research phase. Cheap production. Posttranslational modifications exist. Oral delivery system for antigens by edible plants. Doses inexact.	Mason and Arntzen, 1995
Mammalian cells	Posttranslational modifications, such as glycosylation, phosphorylation and addition of fatty acid chains. Expensive to cultivate. Transient expression relatively fast to achieve, suitable for small amounts of protein. Stable production cell lines laborious but give higher yields.	Geisse et al., 1996
Transgenic animals	Mammary gland expression of interest. Daily protein output. Easy access to the protein. Posttranslational modifications suitable for therapeutic proteins. Costly and tedious research phase. Probably too costly for vaccine production.	Echelard, 1996

Table 4
Selected examples of fusion partners and their effects on the target immunogens[a]

Effector function	Fusion partner	References
Immunopotentiating or carrier-related or properties	Cholera toxin subunit B (CTB)	Dertzbaugh and Elson, 1993; Holmgren et al., 1994; Sun et al., 1994; Cheng-hua et al., 1995; Zhang et al., 1995
	E. coli heat-labile toxin (LTB)	Schödel et al., 1991; Smerdou et al., 1996
	BB or ABP of streptococcal protein G (SPG)	Sjölander et al., 1997; Libon et al., 1999
Targeting	A2 subunit of cholera toxin	Sultan et al., 1998
Increased half-life	B2A3 region of SPG	Makrides et al., 1996
	BB of SPG	Nygren et al., 1991
	IgG Fc	Capon et al., 1989

[a] Fc, fragment crystallizable; IgG, immunoglobulin G.

et al., 1993; Nagahama et al., 1996) are a few examples of bacterial hosts which have successfully been used for antigen production. One potential drawback with prokaryotes as production hosts is that they are unable to carry out post-translational modifications but other beneficial properties, and in particular the cost-efficient production systems, make bacteria the dominating hosts for production of subunit vaccine candidates.

Many different eukaryotic expression systems are available (Geisse et al., 1996), ranging from the simple and cheap yeasts (Sudbery, 1996) to the mammalian cell lines (Geisse et al., 1996) and transgenic animals (Echelard, 1996). Recently, plants have attracted attention as combined production hosts and oral vaccine delivery systems (Mason and Arntzen, 1995). Hepatitis B surface antigen (Mason et al., 1992) and Norwalk virus coat protein (Mason et al., 1996) were expressed in tobacco and potato plants, and assembled into virus-like particles, which were shown to be immunogenic (Thanavala et al., 1995). Oral immunisation, in this case feeding, with transgenic potatoes containing *E. coli* heat labile toxin subunit B (LTB) and the corresponding cholera toxin B subunit (CTB) elicited serum and local antibody responses in mice (Haq et al., 1995; Arakawa et al., 1998), and human clinical trials have been performed with the LTB-expressing potatoes (Tacket et al., 1998).

2.5. Fusion proteins

Genetic fusions can be used to obtain chimeric antigens, into which certain desired properties derived from the fusion partner are added to the target antigen (see Uhlén et al., 1992; LaVallie and McCoy, 1995; Nilsson et al., 1997 for reviews) (Table 4). Producing antigens as fusion proteins is a way of avoiding chemical coupling steps, which might modify the antigen and often result in a heterogeneous antigen preparations. Fusion partners can also simplify the recovery of the protein produced, for example by the introduction of affinity fusion partners, enabling purification by affinity chromatography (Nygren et al., 1994; Nilsson et al., 1997). Other common properties that could be added through gene fusion to thereby simplify the recovery process would include: fusion to a secretion signal to achieve secretion out from the host cell, preferably to the culture medium (Moks et al., 1987; Hansson et al., 1994), or fusion to highly soluble fusion partners to increase the overall solubility of the gene product to simplify recovery and renaturation processes (Samuelsson et al., 1991, 1994).

In this context, an expanded bed adsorption procedure was used in an integrated process for production of a malaria vaccine candidate (Hansson et al., 1994). Efficient recovery of a secreted recombinant fusion protein was achieved,

directly from a crude fermentor broth without prior cell removal. The fusion protein was designed to have a relatively low isoelectric point [p*I*] which allowed anionic exchange adsorption at pH 5.5 at which most *E. coli* host proteins are not adsorbed. This strategy allowed an integration of the cell separation step with ion exchange adsorption of the gene product with simultaneous volume reduction, which resulted in a highly condensed but still efficient recovery process. The two-step purification process, ion exchange chromatography in an expanded bed format followed by IgG affinity chromatography for polishing, demonstrated an overall yield of more than 90% (Hansson et al., 1994).

When required, affinity handles can easily be efficiently removed by site-specific enzymatic cleavage after affinity purification of the fusion protein if cleavage sites are engineered between the target protein and the affinity fusion partner (Nilsson et al., 1997; Jonasson et al., 1998). Of particular interest in this context is the use of affinity-tagged proteases (Gräslund et al., 1997) for efficient removal of the protease after cleavage. However, in other cases it might be advantageous to keep the tag used for affinity capture since it could have positive carrier-related properties (Sjölander et al., 1993, 1997).

Targeting of chimeric antigens to immunoreactive sites can be achieved if using, for example, adhesion factors, monoclonal antibodies or other molecules capable of specifically binding to eukaryotic cell receptors or polysaccharides. The cholera toxin B subunit (CTB) has been extensively investigated as fusion partner to various antigens for its immunopotentiating properties and the capacity of binding to ganglioside GM1 present on mucosal epithelial cells, for targeting mucosal vaccines (Hajishengallis et al., 1995; Liljeqvist et al., 1997a,b). Intracellular targeting of antigens is an elegant variation of the theme. Fusions to the N-terminal catalytic domain of adenylate cyclase toxin (CyaA) of *Bordetella pertussis* resulted in delivery of the foreign viral epitope to the cytosol of cells, by the detoxified invasive toxin. Protective CTL response against challenge with intracerebral lymphocytic choriomeningitis virus (LCMV) challenge was obtained by this targeting strategy (Saron et al., 1997). Chimeric composite immunogens can also be created by fusion of different antigens, such as the hybrid CTB-LTB molecules, which are candidate oral vaccines against both enterotoxic *E. coli* (ETEC) infections and cholera (Lebens et al., 1996). Enhanced immunogenicity was achieved by genetically multimerised T-cell epitopes (Kjerrulf et al., 1997), and genetic combination of B- and T-cell epitopes in a fusion protein has been reported to efficiently simulate both arms of the immune system (Löwenadler and Lycke, 1994).

As a relevant example for the use of fusion proteins in subunit vaccine development, the serum albumin binding region (BB) (Nygren et al., 1988) of streptococcal protein G (SPG) has been shown to have inherent immunopotentiating properties, when used as a carrier protein genetically fused to the immunogen used for immunization (Sjölander et al., 1993, 1997; Power et al., 1997; Libon et al., 1999). It was demonstrated that a fusion protein (BB-M3) containing a malaria peptide, induced significant antibody responses in mice strains that were non-responders to the malaria peptide (M3) alone, suggesting that BB has the ability to provide T-cell help for antibody production (Sjölander et al., 1997). Furthermore, a fusion protein BB-G2N, comprising a 101 amino acid sequence from human RSV, was shown to induce protective immunity in mice to RSV challenge (Power et al., 1997; Libon et al., 1999). It was shown that by inclusion of the BB part, a more potent G2N-specific B-cell memory response was evoked (Libon et al., 1999). This indicates that the SPG-derived BB can function both as an affinity tag to facilitate purification and as a carrier protein with immunopotentiating properties. To date, it is not fully elucidated whether this capacity is due to strong T-cell epitopes (Sjölander et al., 1997) or related to the serum albumin binding activity resulting in a prolonged exposure (Makrides et al., 1996) of the immunogen to the immune system, or a combination of both.

As a conclusion, recombinant techniques are valuable tools for the engineering of fusion proteins, intended for vaccination purposes. Properties useful for the future vaccine antigen, such as

the addition of carrier-related properties, ability to increase the half-life, and possible targeting to special cells or receptors, can be added to the antigen by safe and well-defined methods. In addition, production and purification processes of recombinant antigens can be greatly improved by the use of fusion partners.

3. Live vaccine delivery systems

Live vectors for delivery of heterologous subunit antigens offer a number of advantages as a vaccination strategy. Both Gram-negative and Gram-positive bacteria, including mycobacterial strains, as well as a whole range of viruses, have been investigated for delivery of foreign antigens. The current knowledge of molecular biology and genetics has allowed the development of new attenuation strategies, giving genetically defined attenuated strains of bacteria and viruses, which can be used as carriers for heterologous antigens. By recombinant DNA techniques, the genes encoding the antigens to be delivered can be inserted into the non-pathogenic or attenuated carrier for tailored expression of the subunit vaccine antigens. Strategies employing either chromosomal insertion of the foreign gene or introduction of plasmids into bacteria are being investigated.

Many infectious diseases are caused by pathogens residing at, or entering at, the mucosal site. To induce protection against such pathogens or their toxins, it has been found that, as a complement to systemic immune response, the contribution of the local mucosal response is of great importance (McGhee et al., 1992). The use of live bacteria as mucosal vaccines has been extensively studied, both against the corresponding disease, or as delivery systems for heterologous diseases. Mucosal vaccines are easy to administer, e.g. by the oral or nasal route, and using bacteria as delivery vehicle, the vaccine is comparatively inexpensive to produce (Staats et al., 1994).

A highly interesting feature of genetically engineered bacterial vaccines is the possibility to express the antigen on the surface of the bacterium, for the efficient presentation of the antigen to the cells of the mucosal immune system. Heterologous cell-surface display in the context of live vaccines was first described for Gram-negative bacteria (Georgiou et al., 1993), but Gram-positive and mycobacteria are currently also being investigated for this purpose (Fischetti et al., 1996; Georgiou et al., 1997; Ståhl and Uhlén, 1997). Proteins have also been immobilised on the surface of yeast cells (Schreuder et al., 1996), and in several viral systems, the expression of heterologous virus epitopes on the surface of virus particles has been shown to be advantageous for eliciting immune responses (Dalsgaard et al., 1997) or for enabling targeting of the recombinant virus particles (Ohno et al., 1997). The importance of having the foreign antigen on the surface of the vaccine vector has been extensively debated (Leclerc et al., 1991; Wick et al., 1993; Haddad et al., 1995), but for Gram-positive bacteria, surface display of the antigen seems to be beneficial (Nguyen et al., 1995).

Genetic modification of viruses has enabled the construction of viral subunit vaccines. Highly attenuated recombinant viral vectors, with the possibility of harbouring large foreign genes, are interesting vaccine candidates which may simultaneously elicit immune response to multiple heterologous antigens (see below).

3.1. Gram-negative bacteria

Live recombinant Gram-negative bacteria have been studied in animal models and humans as potential vaccine delivery systems for heterologous antigens. Attenuated pathogenic bacteria can elicit strong and long-lasting immune responses to the foreign antigenic epitopes, by establishing limited infections which resemble the early stages of the natural infections and lead to the induction of natural immune response reactions in the host animal. Since the bacteria are able to survive for some time in the host, the immune responses can be prolonged. Outer surface components, for example lipopolysaccharides (LPS), are natural adjuvants of Gram-negative bacteria to surface-anchored polypeptides.

Shigella flexneri (Ryd et al., 1992; Klee et al., 1997), *V. cholerae* (Schödel et al., 1991; Acheson et al., 1996; Viret et al., 1996; Lång and K

rhonen, 1997), *Yersinia enterocolitica* (Sory et al., 1990), and *B. pertussis* (Mielcarek et al., 1998) are four examples of recombinant attenuated bacteria which have been evaluated as carriers for foreign antigens, but most work on Gram-negative bacteria as vaccine vectors has been done on *E. coli* and *Salmonella* ssp.

3.1.1. E. coli

The Gram-negative enteric bacterium *E. coli* is a potential live vaccine candidate. When given orally, as a mucosal vaccine, undesired immune responses to the vector *E. coli* are not likely to be elicited at high levels, since the bacterium is a natural inhabitant of the human intestine. Although immunisation with *E. coli* expressing viral epitopes as periplasmic fusion proteins has elicited antibodies in mice (Leclerc et al., 1990), surface display of the epitopes is preferable with respect to induced immune responses (Leclerc et al., 1991). The general opinion is that surface display of antigens is favourable for the induction of immune responses when delivering live bacterial vaccines, especially those bacteria which are not capable of invading the mucosal epithelium (Georgiou et al., 1993), but some diverging opinions have been presented (Wick et al., 1993).

Since the first studies on heterologous surface display on *E. coli* were reported in 1986 (Charbit et al., 1986; Freudl et al., 1986), the possibilities of expressing foreign antigens have been thoroughly investigated for *E. coli* (Francisco and Georgiou, 1994). Surface display of antigenic epitopes, antigens, or model antigens has been achieved by insertion of foreign genes into genes encoding *E. coli* outer membrane proteins (Freudl et al., 1986), lipoproteins, or proteins of cellular appendices such as fimbria proteins (Van Die et al., 1988; Hedegaard and Klemm, 1989; Pallesen et al., 1995), pili proteins (Steidler et al., 1993) and the flagellar protein flagellin (Kuwajima et al., 1988); see Ståhl and Uhlén (1997) for a review. Selected examples of successful surface exposure of antigens are listed in Table 5. Some surface expression systems require a mechanism involving several steps for the translocation and surface anchoring of the protein. The *Neisseria gonorrhoeae* IgA protease forms a pore when inserted into the outer membrane, and antigens genetically fused to

Table 5
Examples of *E. coli* surface-display systems[a]

Carrier	Displayed protein	References
LamB	C3 epitope of poliovirus	Charbit et al., 1986
	preS2 region of HBV	Charbit et al., 1987
PhoE	VP1 of FMDV	Agterberg et al., 1990a,b
	hsp65 of *M. tuberculosis*	Janssen et al., 1994
OmpA	Synthetic polylinker	Freudl, 1989
	P. falciparum antigens	Schorr et al., 1991a
Shigella OmpA	VP1 of FMDV	Ruppert et al., 1994
TraT lipoprotein	C3 epitope of poliovirus	Harrison et al., 1990
Lpp-OmpA	β-Lactamase	Francisco et al., 1992; Georgiou et al., 1996
Pseudomonas OprF	vp72 capsid protein of ASFV, gp63 of *Leishmania major*, pre-S2b-epitope of HBV	Cornelis et al., 1996
Neisseria IgAβ	CTB	Klauser et al., 1990, 1992
Flagellin	Hen-egg lysozyme epitope	Kuwajima et al., 1988
FimA	Epitopes from HBV, FMDV, and poliovirus	Hedegaard and Klemm, 1989
FimH	preS2 region of HBV, CTB epitope	Pallesen et al., 1995
P fimbrillin	Epitope from FMDV	van Die et al., 1988
AIDA-I	CTB	Maurer et al., 1997
Klebsiella PulA	β-Lactamase	Kornacker and Pugsley 1990

[a] AIDA-I, adhesin involved in diffuse adherence; ASFV, African swine fever virus; CTB, cholera toxin B subunit; FMDV, foot-and-mouth-disease virus; HBV, hepatitis B virus.

the N-terminus are translocated through the pore (Pohlner et al., 1987; Klauser et al., 1990). Pullulanase of *Klebsiella pneumoniae* is transiently anchored on the surface, and later released into the culture medium (Kornacker and Pugsley, 1990). Antigens fused to pullulanase are ultimately released from the cell when the cells enter stationary growth phase. A chimeric surface-expression system consisting of the signal sequence and the first nine amino acids of *E. coli* major lipoprotein (Lpp) fused to a transmembrane domain from the outer membrane protein A (OmpA) allows for the surface display of large proteins, fused C-terminally of the OmpA (Francisco et al., 1992; Francisco and Georgiou, 1994). Despite the extensive list of different surface display systems developed for *E. coli*, and tested for immunogenicity of exposed antigen, *E. coli* is still considered merely as an experimental live vector.

3.1.2. Salmonella

S. typhi is the cause of severe bacteremia in humans, known as typhoid fever (Gaines et al., 1968). The bacteria colonise the intestinal tract and proliferate in the gut-associated lymphoid tissue (GALT) before they spread systemically to liver and spleen. In mice, the enteropathogenic bacterium *S. typhimurium* is the cause of similar symptoms, by identical mechanisms (Collins, 1972). *Salmonella* spp. have been extensively tested as bacterial vaccine vectors in animal models and in humans. All clinical and field trials with *Salmonella* as delivery system have been limited to attenuated strains of *S. typhi* (Levine et al., 1990). A promising alternative to *S. typhi* as vaccine vector, is the use of attenuated *S. typhimurium* for heterologous antigen delivery.

The licensed oral vaccine strain of *S. typhi*, Ty21, used for vaccination against typhoid fever, was developed in the early 1970s by chemical mutagenesis (Germanier and Furer, 1975). Since then, several strategies of genetically attenuating *Salmonella* have been investigated (Curtiss et al., 1989). Auxotrophic mutants, such as the *aro* mutants with defined mutations in the pre-chorismate biosynthetic pathway, have been constructed of *S. typhimurium* and *S. typhi*. Different *S. typhimurium aro* mutants have proven to be highly attenuated excellent single dose oral vaccines in mice studies (Hoiseth and Stocker, 1981; O'Callaghan et al., 1988), and *aro* mutants of *S. typhi* have been investigated for immunogenicity and safety in human trials (Hone et al., 1991; Tacket et al., 1992, 1997). Regulatory genes, such as the adenylate cyclase (cya) and the cAMP receptor protein (crp) genes, are other targets for mutations in order to achieve attenuated *Salmonella* strains (Curtiss and Kelly, 1987). *S. typhimurium* strains with reduced ability to survive in macrophages were constructed by mutating the *PhoP*, *PhoQ*, or *htrA* genes (Galán and Curtiss, 1989; Johnson et al., 1991). Non-attenuated *S. typhimurium* invades the murine Peyer's patches by selective adherence to the surfaces of M cells, followed by invasion and killing of the invaded cells, which enables the bacteria to get access to the underlying mucosa (Clark et al., 1998). Some attenuated strains enter M cells and dendritic cells, but leave them intact, making attenuated *Salmonella* highly suitable as recombinant vaccine vectors, presenting expressed foreign antigen to the mucosal immune system (Hopkins and Kraehenbuhl, 1997; Dustan et al., 1998).

Numerous studies of attenuated *Salmonella* as live vectors expressing heterologous antigens have been performed for development of oral vaccines against bacterial, viral, or parasitic diseases (Hackett, 1993; Chatfield et al., 1995; Georgiou et al., 1997). Live *Salmonella*, being an intracellular pathogen, is generally capable of eliciting cellular immune responses to the antigen delivered, a desired property of the immune responses protecting against viral or parasitic diseases.

Mucosal immunisation of mice with recombinant *S. typhimurium* expressing bacterial antigens, such as pertussis toxin-derived antigens (Walker et al., 1992; Anderson et al., 1996; Pozza et al., 1998), and tetanus toxin antigens (Fairweather et al., 1990; Chatfield et al., 1992), have elicited specific antibody responses. Viral antigens, derived from for example Hepatitis B virus (Wu et al., 1989; Schödel et al., 1990), herpes simplex virus (Karem et al., 1997), influenza virus (Rüssmann et al., 1998), and transmissible gastroenteritis virus (Smerdou et al., 1996), have elicited antibody responses in mice when delivered by the

live vaccine vector *S. typhimurium*. Hepatitis B virus core antigen (Hopkins et al., 1995) and human papillomavirus type 16 capsid protein (Nardelli-Haefliger et al., 1997) are expressed and assemble into virus-like particles in *Salmonella*. Mucosal immunisation of mice with such recombinant *S. typhimurium* cells induces immunity to the viral proteins. Protective immune responses have been obtained in mice using *S. typhimurium* vector delivering *Shistosoma mansoni* (Khan et al., 1994) or *Plasmodium berghei* (Sadoff et al., 1988) antigens. Mice were protected from *Helicobacter pylori* infection by intranasal immunisation with live recombinant *S. typhimurium* expressing the urease A and B subunits (Corthésy-Theulaz et al., 1998). The presence of IgG1 and IgG2A demonstrated the generation of both Th1 and Th2 type of response (Corthésy-Theulaz et al., 1998).

Many of the *E. coli* surface expression systems for the delivery of heterologous antigens to immunised animals, have been used for the surface-display of immunogens in *Salmonella* live vaccine candidates (Georgiou et al., 1997; Ståhl and Uhlén, 1997). For example, malarial antigens have been surface exposed on *S. typhimurium* by genetic fusion to the *OmpA* gene (Schorr et al., 1991b; Haddad et al., 1995), and fusions to the *E. coli* LamB and MalE proteins have been used for the surface expression or periplasmic targeting, respectively, of viral antigens (Charbit et al., 1993) and the Shiga toxin B subunit (Su et al., 1992). Shiga-like toxin IIe was exported by the use of an *E. coli* hemolysin transport system and fusion to the outer membrane protein TolC (Tzschaschel et al., 1996). Also naturally existing *Salmonella* surface proteins or appendices have been used for surface display (Newton et al., 1989; Wu et al., 1989; McEwen et al., 1992).

Despite extensive research efforts and the existence of numerous expression systems, the early optimism of using *Salmonella* spp. as delivery systems for foreign antigens has been somewhat hampered. The main reason is the risk of side-effects due to potential reversion into virulence in immunocompromised humans, and lack of efficiency in human trials. Nevertheless, *Salmonella* provides an excellent research tool as experimental vaccines, stimulating both the humoral and cellular branch of the immune system.

3.2. Gram-positive bacteria

Gram-positive bacteria have also been investigated for delivery of heterologous antigens for vaccine purposes. For example, attenuated pathogenic bacteria, such as the mycobacterial vaccination strain bacille Calmette-Guérin (BCG) and *Staphylococcus aureus*, have been studied, but recently the use of non-pathogenic food grade or commensal bacteria have attracted significant attention. Non-pathogenic bacteria are safe to use for immunisation, since there is no risk of reversion to virulence. For commensal and food-grade bacteria, it is unlikely that a strong undesired immune response to the vector is evoked.

Gram-positive expression systems for cytoplasmically expressed or secreted antigens have been developed (Iwaki et al., 1990; Wells et al., 1993a,b). However, the use of surface display systems for Gram-positive vaccine vectors has been extensively studied for several bacteria in a number of disease models (see Table 6 for selected examples) and has been considered advantageous for eliciting mucosal immunity when using Gram-positive bacteria as vaccine delivery system (Nguyen et al., 1995; Fischetti et al., 1996; Ståhl and Uhlén, 1997).

Many surface proteins of Gram-positive bacteria anchor via their carboxy termini through a conserved mechanism of surface attachment. The mechanism was elucidated by Schneewind and colleagues, who studied the sorting of *S. aureus* protein A (SpA) to the cell wall (Fischetti et al., 1990; Schneewind et al., 1992, 1993, 1995; Navarre and Schneewind, 1994; Ton-That et al., 1997). The C-terminal region which consists of a charged repetitive region, a highly conserved enzymatic cleavage site, a hydrophobic anchor and a charged tail (Fig. 1), is responsible for the surface attachment. The repetitive region of charged amino acids, thought to interact with the peptidoglycan cell wall (Guss et al., 1984), and the hydrophobic region of 15–20 amino acids, enough to span the cytoplasmic membrane, are separated with a highly conserved LPXTG motif.

Table 6
Selected examples of surface display of heterologous protein antigens on Gram-positive bacteria[a]

Bacterium	Surface-display system	Displayed proteins	References
Food-fermenting bacteria			
S. xylosus	*S. aureus* protein A	Epitope of glycoprotein G of RSV	Hansson et al., 1992; Nguyen et al., 1993, 1995; Robert et al., 1996
S. carnosus	*S. aureus* protein A	Epitope of glycoprotein G of RSV	Samuelson et al., 1995; Robert et al., 1996; Ståhl et al., 1997
L. lactis	*L. lactis* proteinase PrtP	Tetanus toxin fragment C	Wells et al., 1993a; Norton et al., 1996, 1997; Robinson et al., 1997
Lactobacillus paracasei	*S. pyogenes* protein M6	gp41 of HIV-1	Rush et al., 1997
Commensal bacteria			
S. gordinii	*S. pyogenes* protein M6	Allergen Ag5.2 from white-faced hornet	Medaglini et al., 1995
		E7 protein of HPV 16	Oggioni et al., 1995; Medaglini et al., 1997; Di Fabio et al., 1998
		gp120 of HIV-1	Pozzi et al., 1994; Di Fabio et al., 1998
Other bacteria			
B. subtilis	*B. subtilis* CwbA	*Y. pseudo-tuberculosis* invasin	Acheson et al., 1997
Mycobacterium bovis	*M. tuberculosis* membrane-associated lipoprotein	*Borrelia burgdorferi* OspA	Stover et al., 1993

[a] CwbA, cell-wall-bound autolysin modifier protein; HIV, human immunodeficiency virus; HPV, human papilloma virus; OspA, outer surface protein A; RSV, respiratory syncytial virus.

Proteolytic cleavage occurs between the threonine and glycine residues within the LPXTG motif, after which the surface protein is covalently linked to the cell wall (Schneewind et al., 1995; Ton-That et al., 1997).

Surface display expression systems developed for *Staphylococcus xylosus* (Hansson et al., 1992) and *Staphylococcus carnosus* (Samuelson et al., 1995) employ the C-terminal cell surface-anchoring regions of SpA. The heterologous staphylococcal fusion proteins are expressed from plasmids, while the *Streptococcus gordinii* surface-display system (Pozzi et al., 1992) is based on chromosomal integration of a gene fusion between the *Streptococcus pyogenes* surface protein M and a foreign antigen (Pozzi et al., 1992). The protein M was successfully surface displayed on various lactic acid bacteria (Piard et al., 1997), suggesting the applicability of the corresponding recombinant surface display system to those bacteria. Recombinant proteins were targeted to the cell surface in *Lactococcus lactis* by fusion to the C-terminal region of proteinase PrtP, a cell surface-associated enzyme (Norton et al., 1996). It should however be noted that PrtP is membrane-associated, which is why the expressed antigen is not accessible at the outer cell-surface. Surface expression of foreign antigens on recombinant bacille Calmette-Guérin (BCG) was achieved by

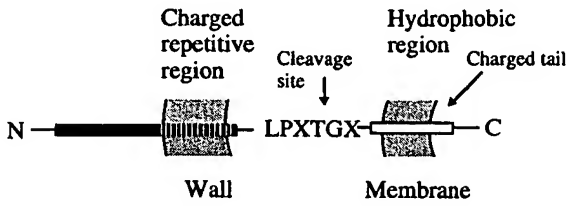

Fig. 1. Schematic representation of the C-terminal region of *S. aureus* protein A, which has been used extensively for surface display applications on Gram-positive bacteria. The translocation and anchoring process involves cleavage within the LPXTG motif.

fusion of the antigen-encoding gene to the gene for the membrane-associated lipoprotein of *Mycobacterium tuberculosis* (Stover et al., 1993). BCG is one of the most promising live vectors besides the food-grade and commensal bacteria (Stover et al., 1991; O'Donnell, 1997). Since BCG is an intracellular pathogen, in analogy with the Gram-negative *Salmonella* spp., it is often possible to obtain cellular immune responses to antigens delivered by BCG. The candidate lyme disease vaccine developed by Stover et al. (1993) is currently being evaluated in clinical trials. Fusion to the cell wall-bound autolysin modifier protein CwbA was used for the surface expression of *Yersinia pseudotuberculosis* invasin in *Bacillus subtilis* for the construction of a heat-stable spore-based vaccine candidate (Acheson et al., 1997).

Gram-positive commensal or food-fermenting bacteria are highly interesting candidates for mucosal vaccines, as demonstrated by the numerous successful immunisation studies in which specific antibody responses were elicited to surface exposed foreign antigens (Fischetti et al., 1996; Ståhl and Uhlén, 1997). The human commensal *S. gordinii*, has been extensively studied as recombinant vaccine vector, displaying antigens derived from HIV (Pozzi et al., 1994; Di Fabio et al., 1998), human papilloma virus (Oggioni et al., 1995; Medaglini et al., 1997), and the allergen Ag5.2 of the white-faced hornet (Medaglini et al., 1995). Mucosal immunisation of mice and monkeys elicited antigen-specific IgA and IgG (Medaglini et al., 1995, 1997; Oggioni et al., 1995; Di Fabio et al., 1998), suggesting that the *S. gordinii* delivery system could become suitable as mucosal vaccine-delivery system.

Oral immunisation of mice with *S. xylosus* cells displaying hybrid surface proteins containing a trimerised 14 amino acid epitope derived from human respiratory syncytial virus (RSV) attachment glycoprotein (G protein) resulted in specific serum IgG-antibodies (Nguyen et al., 1993). Using ABP as a surface-accessible model antigen, *S. carnosus* was compared to *S. xylosus* as potential live vaccine delivery vehicles (Ståhl et al., 1997), and a higher titer anti-ABP antibodies was found in the mice immunised orally with the *S. carnosus* cells. The higher surface density of the recombinant receptors on *S. carnosus* (Robert et al., 1996; Andréoni et al., 1997) may account for the higher antibody responses observed for these cells. The importance of the surface localization of foreign antigens has been investigated in several studies. In a comparative study, mice were immunised subcutaneously with live recombinant *S. xylosus* cells, expressing the foreign antigen on the surface of the bacteria or intracellularly (Nguyen et al., 1995). No specific antibodies were detected when the antigen was expressed in a non-secretable form by the live vectors, whereas the bacteria carrying surface-exposed antigens elicited antibody responses to the heterologous antigen upon immunisation. Similar observations were made with recombinant BCG (Stover et al., 1993) and with *L. lactis* for surface-associated antigen (Norton et al., 1996), thus stressing the importance of surface accessibility of the foreign antigen.

Surface expression on Gram-positive cells was also investigated for application areas other than vaccine development. Microbial biocatalysts can be envisioned by the surface expression of active enzymes on *S. carnosus* (Strauss and Götz, 1996), and the surface display of an active human IgE-binding single chain antibody demonstrates the possibility of developing diagnostic tools based on Gram-positive cells (Gunneriusson et al., 1996). However, these and other applications will not be further discussed since they fall outside the scope of this review.

3.3. Viral delivery systems

Recombinant viruses have been studied as candidate vaccines, both as vaccines against the original diseases, or as viral vectors delivering heterologous antigens or genes. Viruses have also been used in cancer immunotherapy models (Restifo, 1996) and in gene therapy (Robbins et al., 1998). Many different viruses have been investigated as potential recombinant vaccine vectors against various infectious diseases (see Table 7 for selected examples). Viral vaccines have the advantageous ability to elicit both humoral and cellular immune responses towards the target antigen, because of the intracellular expression of the heterologous antigens, leading to presentation by the MHC class I molecules.

Table 7
Selected examples of viral vectors and delivered heterologous antigens[a]

Viral vector	Antigen	References
Vaccinia	HIV-1 glycoprotein	Katz et al., 1997
	Leishmania antigen	McMahon-Pratt et al., 1993
	Malarial epitopes	Miyahira et al., 1998
NYVAC	Malarial antigens	Tine et al., 1996
	HIV-1 glycoprotein	Cox et al., 1993b
MVA	Influenza antigen	Bender et al., 1996
	SIV antigens	Hirsch et al., 1996
ALVAC	Rabies virus glycoprotein	Cadoz et al., 1992
	HIV multiple antigens	Myagkikh et al., 1996
Adenovirus	Measles virus nucleocapsid	Fooks et al., 1995
	Rabies virus glycoprotein	Xiang et al., 1996
Semliki Forest Virus	Influenza nucleoprotein	Zhou et al., 1995
	HIV gp160	Berglund et al., 1997
Sindbis	Japanese encephalitis virus antigens	Pugachev et al., 1995
VEE	Influenza hemagglutinin	Davis et al., 1996b
Influenza	Epitope of HIV-1 gp41	Muster et al., 1995
Polio	HBV antigens	Yim et al., 1996
	SIV antigens	Anderson et al., 1997
Mengo	HIV gp120	Altmeyer et al., 1994

[a] ALVAC, attenuated canarypox virus strain; HIV, human immunodeficiency virus; MVA, modified vaccinia virus Ankara strain; NYVAC, attenuated vaccinia virus strain; SIV, Simian immunodeficiency virus; VEE, Venezuelan equine encephalitis virus.

Vaccinia virus was earlier used in vaccination against variola virus, a vaccination campaign leading to the eradication of smallpox in 1979. Since 1982, when introduction of genes encoding foreign proteins into vaccinia was first demonstrated (Mackett et al., 1982; Panicali and Paoletti, 1982), vaccinia virus has been extensively studied as live vaccine vector (Ulaeto and Hruby, 1994). Recombinant vaccinia virus was originally produced by homologous recombination, where cells are transfected with a plasmid containing the foreign gene flanked by regions identical to sequences in the vaccinia genome. After the infection of the cells with vaccinia virus, the recombination occurred. This is still the most common method for introducing heterologous genes in vaccinia, although alternative strategies have been developed (Carroll and Moss, 1997).

Immunisation experiments with vaccinia recombinants expressing viral (Smith et al., 1983; Moss et al., 1984), bacterial (Fischetti et al., 1989; Iacono-Connors et al., 1991), and parasitic (McMahon-Pratt et al., 1993) antigens have been reported to elicit protective immunity in animal disease models. An oral rabies vaccine consisting of recombinant vaccinia expressing the rabies virus glycoprotein (Brochier et al., 1991) has been given to wild animals, leading to steep decrease in the incidence of rabies in Belgium (Brochier et al., 1995). The surface display concept, only recently investigated for recombinant vaccinia, where a chimeric HIV-1 glycoprotein was targeted to the outer envelope of vaccinia (Katz et al., 1997), was also demonstrated to be favourable for viral vectors, in terms of immunogenicity of the heterologous antigen (Katz and Moss, 1997).

Due to safety concerns, recombinant vaccinia virus based on wild-type vaccinia is primarily investigated as veterinary vaccines. For human use, highly attenuated, non-replicative, vaccinia vectors have been constructed, for example the NYVAC strain, with deletion of 18 open-reading frames from the original genome (Tartaglia et al., 1992; Paoletti, 1996), and the modified vaccinia virus Ankara strain (MVA) (Sutter and Moss, 1992). Recombinant NYVAC and MVA vectors encoding a wide range of pathogen-derived antigens have been tested in viral, bacterial, and parasitic disease models (Perkus et al., 1995; Carroll and Moss, 1997). Other poxviruses have also been used as vaccine vectors, e.g. racoonpox (Esposito et al., 1988; Hu et al., 1996), capripox (Romero et al., 1994) and the avipox viruses. Among these, fowlpox (Ogawa et al., 1990) and canarypox viruses, lacking replication ability in mammalian cells, have been extensively investi-

gated. ALVAC, a recombinant canarypox virus, is the most studied non-human poxvirus used for delivery of heterologous antigens (Perkus et al., 1995).

Adenoviral vaccine vectors are not pathogenic in humans, can be made replication competent or deficient, and can be administered orally (Imler, 1995). Humoral (Fooks et al., 1995), cell-mediated (Xiang et al., 1996) and mucosal (Mittal et al., 1996) immunity can be elicited to the heterologous antigens delivered by recombinant adenoviruses. Several viral antigens, for example the HBV surface antigen (Lubeck et al., 1989; Chengalvala et al., 1997), the measles virus nucleocapsid (Fooks et al., 1995), and glycoproteins from the herpes simplex virus (Gallichan et al., 1993) and rabies virus (Xiang et al., 1996), have been expressed and delivered by adenoviral vectors. Self-assembled virus-like particles, which are not infectious, have also been used as delivery systems for heterologous antigens. Fusion of a viral epitope to the N-terminus of the VP2 capsid protein of parvovirus led to the assembly of parvovirus-like particles, which were able to deliver the foreign CTL epitopes into the cytosol, resulting in stimulated cellular immunity (Sedlik et al., 1997).

Although a number of clinical trials with different viral recombinant vectors have been performed, no such vaccine candidate has progressed beyond phase II evaluation, since the profiles of the immune responses elicited were not considered ideal. Until the safety and immunological questions are solved, only certain specific vaccine niches, such as HIV and cancer, as well as veterinary vaccines, would be the applications for virus-based vectors.

4. Nucleic acid vaccines

A completely new field of vaccination was opened by the pioneering works of Liu and colleagues, who in 1993 reported that direct injections of a gene from influenza A virus induced protective immune response in immunised mice (Ulmer et al., 1993). Since then, the technology of DNA vaccination has become well established and widely spread in the research community as a method for infectious disease prophylactics (Ulmer et al., 1996c; Donnelly et al., 1997).

4.1. DNA

DNA vaccines consist of plasmid DNA expression vectors of *E. coli* origin, which encode the antigen or antigens of interest under the control of strong viral promoters recognised by the mammalian host. When the plasmid DNA is administered to an animal, the antigen is expressed in situ, leading to an antigen-specific immunity. This genetic vaccination method offers a number of attractive qualities: the simplicity of producing large quantities of pure DNA, the breadth of the applicability to various pathogens, the ability to induce cellular immune responses through MHC class I presentation, and the potential to manipulate the immune response through the co-delivery of genes encoding immunologically relevant molecules (Davis, 1997).

The first report of injection of non-replicating DNA plasmids in saline into muscle cells, with the subsequent long-term expression of the reporter genes, was published in 1990 (Wolff et al., 1990), and 2 years later, Tang and co-workers showed the induction of an immune response in mice against plasmid encoded protein human growth hormone (Tang et al., 1992) by bombarding the skin with DNA-coated gold microprojectiles. After the following demonstration of the protective efficacy of immunisation with DNA encoding influenza A virus nucleoprotein (Ulmer et al., 1993), DNA vaccines have been reported to generate immune responses against various antigens, and also protective immunity in several disease models, of which selected examples are given in Table 8.

DNA expression plasmids, delivered either by intramuscular injection in saline preparation, intravenous administration as liposome-DNA complexes (Huang and Li, 1997), intranasally using a bacterial vector (see below) (Sizemore et al., 1997), by oral delivery of microencapsulated DNA (Schubbert et al., 1997), or by high velocity bombardment of DNA-coated particles (Klein and Fitzpatrick-McElligott, 1993), have proven to be advantageous in eliciting cytotoxic T

Table 8
Selected examples of DNA immunisations[a]

Pathogen	DNA encoded antigen	Reference
B. burgdorferi	OspA	Luke et al., 1997
Bovine herpes virus	Glycoproteins gI, gII, gIV	Cox et al., 1993a
Bovine respiratory syncytial virus	G protein	Schrijver et al., 1997
Cytomegalovirus	ppUL83	Pande et al., 1995
Ebola virus	Nucleoprotein, glycoprotein	Xu et al., 1998
Encephalitis virus SLE St. Louis strain	prM/E protein	Phillpotts et al., 1996
Hepatitis B virus[b]	HBV surface antigen HBsAg	Davis et al., 1993, 1996a
	HBsAg	Michel et al., 1995
	HBcAg, HBeAg	Kuhober et al., 1996
	HBsAg	Chow et al., 1997; Gregoriadis et al., 1997
Hepatitis C virus	Nucleocapsid	Major et al., 1995
	Core protein	Chen et al., 1995
Hepatitis E virus	Structural protein ORF-2	He et al., 1997
Herpes simplex virus	Immediate early protein ICP 27	Manickan et al., 1995
	Glycoprotein D	Bourne et al., 1996
	Glycoprotein B	Kuklin et al., 1997
HIV[b]	Glycoprotein gp160	Wang et al., 1993
	env, rev, gag/pol	Boyer et al., 1997
	gag/pol, envelope proteins	Kim et al., 1997b
	gp160, p24, rev, tat, nef	Hinkula et al., 1997
	gp120	Fuller et al., 1997
Influenza A virus[b]	Nucleoprotein (NP)	Ulmer et al., 1993; Yankauckas et al., 1993; Donnelly et al., 1995; Deck et al., 1997
L. major	Major surface glycoprotein gp63	Xu and Liew, 1995
Lymphocytic choriomeningitis virus	Nucleoprotein (NP)	Martins et al., 1995
	NP, Glycoprotein	Yokoyama et al., 1995
Measles virus	Hemagglutinin, NP	Cardoso et al., 1996
M. tuberculosis	Antigen 85 (Ag85)	Huygen et al., 1996
	M. leprae hsp 65	Tascon et al., 1996
Papillomavirus	Major capsid protein L1	Donnelly et al., 1996
Plasmodium yoelii[b]	Circumsporozoite protein (CSP)	Sedegah et al., 1994
	CSP	Mor et al., 1995
	Hepatocyte erythrocyte protein 17	Doolan et al., 1996; Gramzinski et al., 1997
Prion proteins	Cellular prion (PRNP)	Krasemann et al., 1996

Table 8 (*continued*)

Rabies virus	Glycoprotein	Xiang et al., 1994; Ray et al., 1997
Rotavirus	Envelope, Vp4, Vp6, Vp7	Herrmann et al., 1996
Schistoma japonicum	Paramyosin, Sj97	Yang et al., 1995; Waine et al., 1997
Simian immunodeficiency virus	Env, Gag	Lu et al., 1997

[a] CSP, circumsporozoite protein; HBcAg, hepatitis B virus core antigen; HBsAg, hepatitis B virus surface antigen; NP, nucleoprotein; ORF, open reading frame.

[b] Clinical trials ongoing.

lymphocyte (CTL) response, and T helper (Th) responses, as well as humoral immunity to encoded antigens, often being of viral or parasitic origin (Pardoll and Beckerleg, 1995; Ulmer et al., 1996b).

The immune responses to DNA vaccines can be enhanced by the DNA acting as its own adjuvant. Immunostimulatory properties have been attributed to sequences with a cytosine preceding a guanosine (CpG motif) (Sato et al., 1996). Unmethylated CpG dinucleotide motifs with certain flanking regions have been found to stimulate several types of immune cells in vitro (Krieg et al., 1995; Ballas et al., 1996). In vivo, such motifs have acted as a Th1 enhancing adjuvant (Cowdery et al., 1996; Chu et al., 1997; Weiner et al., 1997; Davis et al., 1998) suggesting that they may contribute to the Th1-mediated immune response observed to be dominant after immunisation with DNA of bacterial origin (Klinman et al., 1997; Roman et al., 1997).

Part of the appeal of DNA vaccines is the potential to manipulate the immune response generated upon immunisation. One strategy was the co-delivery of plasmid DNA encoding the T-cell co-stimulatory molecule B7-2, also denoted CD86, which led to increased Th and CTL responses (Kim et al., 1997b). Enhanced Th and CTL responses were also obtained when co-injecting with plasmid encoding IL-12 (Kim et al., 1997a). The antibody response to the DNA vaccine was, as expected, downregulated by this delivery of IL-12 encoding plasmid (Kim et al., 1997a). Xiang and Ertl (1995) observed an upregulation of the antibody response to plasmid-encoded rabies virus glycoprotein in mice by co-inoculation with plasmid encoding the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF). The immune responses to a plasmid-encoded antigen could also be controlled by the method of immunisation, as described by Feltquate and co-workers who, utilising an influenza hemagglutinin (H1)-expressing plasmid, obtained a predominant Th1 response by saline injection of DNA, but using a gene gun DNA immunisation, the Th2 response with IgG1 anti-H1 antibodies dominated (Feltquate et al., 1997). Similar observations were made by immunising with influenza A nucleoprotein encoding DNA (Pertmer et al., 1996). The Th1/Th2 balance may also be affected by the dose of antigen (Barry and Johnston, 1997), or by the cellular location of the antigen (Lewis et al., 1996; Haddad et al., 1998). The target tissues behave differently when transfected by DNA, in terms of Ab and CTL-responsiveness (Torres et al., 1997).

In addition to the use of plasmid DNA for prophylactic vaccination against infectious diseases, DNA vaccines may be useful as treatment of individuals chronically infected with viruses, e.g. HIV and hepatitis virus strains B and C. In a mouse model of a hepatitis B virus chronic carrier, a DNA vaccine was able to break tolerance and downregulate viral gene expression, which demonstrates the potential of immunotherapeutic DNA vaccination (Mancini et al., 1996). An interesting variant of DNA vaccination against pathogens is the somatic transgene immunisation (STI), i.e. the intraspleenic inoculation of plasmid DNA encoding an Ig heavy-chain. The DNA is taken up by B lymphocytes and transgene H-chain immunoglobulins are expressed and secreted, result-

ing in immunity against the transgene product (Gerloni et al., 1997b). By engineering the complementarity-determining regions (CDR) of an antibody to contain specific sequences of antigens, so called antigenized antibodies are obtained, which can mimic the native antigen conformation and target antigen-presenting cells via the Fc receptor (Zanetti, 1992). Using STI, immunising with plasmid DNA encoding antigenised antibodies with different malarial B- and T-cell epitopes, immunity to *Plasmodium falciparum* sporozoites was obtained (Gerloni et al., 1997a; Xiong et al., 1997a). Somatic gene immunisation offers promise for future treatment of genetic disorders, and autoimmune diseases, as well as for the treatment of cancer (see Sandhu et al., 1997 for a review). Suppressive vaccination in a rodent model of multiple sclerosis has been performed immunising with DNA encoding a T-cell receptor V-region (Waisman et al., 1996). DNA vaccination has also been investigated for the treatment of allergies, where an inhibition of specific IgE antibodies after genetic immunisation has been reported (Hsu et al., 1996a; Raz et al., 1996). DNA vaccines encoding SV-40 large tumour antigen (Schirmbeck et al., 1996), variable regions of the light and heavy chains of an idiotypic antibody (Hawkins et al., 1993), and single T-cell epitopes (Ciernik et al., 1996) have proven to be effective in reducing tumours in murine models. DNA-based immunisation has also been used as a research tool, for example for the production of polyclonal and monoclonal antibodies, circumventing the need for production and purification of a recombinant protein (Barry et al., 1994). Taking advantage of the efficiency of DNA cloning techniques, entire expression libraries have been inserted in DNA expression vectors for the identification of immunogenic proteins, based on protection upon immunisation (Barry et al., 1995; Johnston and Barry, 1997; Alberti et al., 1998).

Alphaviruses are single-stranded RNA viruses with a broad host range, which allow infection of a variety of cells (Strauss and Strauss, 1994). The positive RNA genome encodes its own replicase mediating RNA-RNA replication. Infection of cells or transfection of genomic RNA into the cytoplasm of the cell leads to efficient replication, high-level expression of structural proteins, and the assembly of a high number of virus particles. Since the genes for the structural proteins are expressed from a subgenomic RNA, separated in the genome from the replicase gene, manipulation of the subgenomic sequences does not affect the replication (Berglund et al., 1996; Tubulekas et al., 1997). Strategies taking advantage of alphavirus replicase to create an in vivo amplification system to improve DNA vaccine vectors are under investigation. Immunisation studies with recombinant alphaviral DNA vectors have shown the induction of protective immune responses (Berglund et al., 1998). The immunized mice developed more pronounced immune responses than mice that received a conventional DNA vaccine vector. The in vivo expression mediated by the alphaviral vector was found to be transient and lytic, which should be beneficial in terms of biosafety and tolerance-induction concerns (Berglund et al., 1998).

A number of safety questions need to be answered when considering the delivery of DNA to a mammalian host. There is an obvious anxiety that the DNA could become integrated into the host genome, and thereby activate a host protooncogene or deactivate a suppressor gene, and thus induce cancer. The possibility of induction of anti-DNA antibodies upon immunisation of plasmid DNA, in parallel with the immune response desired, must also be considered. However, none of these possible dangers have so far been observed in animal studies. The few events of integration of foreign DNA into host DNA that have been reported, concerned the integration of genes in B lymphocytes, with a persistence of 3–4 months (Xiong et al., 1997b), Ig encoding DNA into spleenocytes (Gerloni et al., 1997b), and plasmid DNA into macrophages, delivered by recombinant *Listeria* (Dietrich et al., 1998). Elucidating the mechanisms for antigen presentation after DNA immunisation (Ulmer et al., 1996a; Fu et al., 1997) and investigating the possibilities of combined DNA/protein regimens (Letvin et al., 1997), will add to the potential of DNA vaccines to elicit qualitative immune response ideal for a given pathogen.

4.2. RNA

Genetic vaccination through the delivery of RNA has also been investigated, but to lesser extent than DNA vaccination. RNA expression is short-lived, and is thus less effective in inducing an immune response. The preparation and administration of RNA is troublesome because of the low stability of the RNA. One advantage of the RNA strategy is that there is no risk of integration of the delivered gene into the host genome. The induction of anti-influenza cytotoxic T lymphocytes (CTL) in vivo by immunising mice with liposome-entrapped mRNA encoding the influenza virus nucleoprotein has been described (Martinon et al., 1993). Liposome-mediated transfection has also been used for the delivery of mRNA encoding human carcinoembryonic antigen (CEA) to mouse fibroblasts (Conry et al., 1995). The rapid decline in anti-CEA antibody levels observed could reflect a short-lived in vivo protein expression encoded by the mRNA transcripts, which could be desirable when delivering genes encoding proto-oncogene products or growth factors, with a risk of malignant transformation as a consequence of prolonged protein expression.

There is a major advantage in using RNA carrying the gene encoding an alphaviral replicase (e.g. the Semliki Forest Virus (SFV) replicase) together with the gene encoding the foreign antigen, compared to the delivery of an mRNA molecule encoding only the antigen. Once such a construct has been transfected to a mammalian cell, the translation machinery produces the viral replicase which starts mass replication of the RNA (carrying the antigen-encoding gene). Protein expression in vivo will thus be very effective for a short period of time, until the antigen-producing cell dies. Alphaviral-based RNA for vaccination purposes can be delivered either as naked RNA orpacked in non-replicative viral particles (Tubulekas et al., 1997). Recombinant SFV particles containing the RNA encoding influenza nucleoprotein induced strong immune responses in mice (Zhou et al., 1995), and were only recently demonstrated to be protective (Berglund et al., 1998). Immune responses to HIV-1 (Berglund et al., 1997) and SIV (Mossman et al., 1996) proteins were elicited when immunising primates with recombinant SFV particles with packed RNA. Even naked recombinant RNA constructs utilizing alphaviral replicase-genes have given in vivo expression of antigen (Johanning et al., 1995) and strong immune responses to those antigens have been observed in mice (Zhou et al., 1994). This strategy could perhaps be an efficacious way of delivering nucleic acid vaccines since it avoids the risk of host chromosomal integration.

4.3. Bacterial plasmid DNA-delivery systems

The use of bacteria as delivery system for antigen-encoding plasmid DNA to mammalian cells has recently been investigated as an alternative to intramuscular or intradermal immunisation of DNA. One advantage of using bacteria is the possibility of using non-parental immunisation routes, and thereby stimulating mucosal immunity. Attenuated *S. flexneri* was used as a DNA delivery vehicle, which invaded mammalian cells and delivered mammalian expression plasmids to the cytoplasm of the infected cells (Sizemore et al., 1995). The expression of plasmid-encoded β-galactosidase was demonstrated in baby hamster kidney (BHK) cells, and intranasal immunisation of mice raised serum antibodies specific for β-galactosidase (Sizemore et al., 1997). Delivery of plasmid DNA encoding model antigens to macrophages was studied in vitro using attenuated *Listeria monocytogenes* as suicide vector (Dietrich et al., 1998). Both efficient expression and antigen presentation was demonstrated. Also demonstrated was integration of plasmid DNA into the macrophage cell's genome, at a rate of 10^{-7}. The intracellular pathogen *S. typhimurium* has also been used for DNA delivery (Darji et al., 1997). Nucleic acid vaccination strategies are of significant interest, and the use of bacterial delivery systems for DNA vaccines, as an alternative to the intramuscular saline injections or gene-gun administration, could make a mucosal DNA vaccine possible, combining the advantages of a bacterial vector and mucosal administration with the simplicity of DNA vaccines.

5. Concluding remarks and future perspectives

Several rational techniques have recently become available in the search for novel target antigens for use in new vaccines. The large-scale genome sequencing projects have provided enormous amounts of information to be deciphered. Vaccinology will most probably benefit through the identification of new target antigens but also through understanding the mechanisms of infection and immunity.

New target antigens can also be identified for evaluation of immunogenicity using expression library immunisation (ELI) (Johnston and Barry, 1997). To create an expression library, the genome of a pathogen is digested into fragments, which are ligated into eukaryotic expression plasmids, forming the total library. By immunising animals with sublibraries, containing a large number of different plasmids, immune responses evoked in the animals are the basis for selection of a sublibrary to be further studied in the identification of the single protective plasmid. Another approach to the identification of target antigens has been envisioned by Jacobsson (1997), who used the method denoted shotgun phage-display for identifying genes encoding binding domains of bacterial adhesins (Jacobsson and Frykberg, 1995, 1998). *S. aureus* chromosomal DNA was fragmented by sonication and inserted into phagemid vectors for expression on phages. After affinity selection of the libraries against various human proteins, ligand-binding phages were identified, containing DNA encoding the bacterial binding protein fragment. Identified fragments of bacterial adhesins could be of interest in a future vaccine as candidate antigens or as fusion partners for vaccine targeting.

In the research area of antigen targeting, proteins binding to a surface receptor or polysaccharide could potentially also be selected from combinatorial libraries based on a protein with a randomised binding surface (Clackson and Wells, 1994; Nygren and Uhlén, 1997). After selection of a binder from the library, this protein can be used as a fusion partner to the antigen, for targeting of the antigen to certain tissues or cell types.

Taken together, it is evident that recombinant DNA technology will have a major impact on future strategies for the prevention of infectious diseases. The design, selection and production of recombinant subunit vaccines will be the basis of modern vaccinology. Various strategies to administer the subunit vaccine, as a protein immunogen, via a live delivery vehicle or as nucleic acid constructs, will be available.

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